

Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro

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Abstract

Experimentally, peripheral nerve repair can be enhanced by Schwann cell transplantation but the clinical application is limited by donor site morbidity and the inability to generate a sufficient number of cells quickly. We have investigated whether adult stem cells, isolated from adipose tissue, can be differentiated into functional Schwann cells. Rat visceral fat was enzymatically digested to yield rapidly proliferating fibroblast-like cells, a proportion of which expressed the mesenchymal stem cell marker, *stro-1*, and *nestin*, a neural progenitor protein. Cells treated with a mixture of glial growth factors (GGF-2, bFGF, PDGF and forskolin) adopted a spindle-like morphology similar to Schwann cells. Immunocytochemical staining and western blotting indicated that the treated cells expressed the glial markers, GFAP, S100 and p75, indicative of differentiation. When co-cultured with NG108-15 motor neuron-like cells, the differentiated stem cells enhanced the number of NG108-15 cells expressing neurites, the number of neurites per cell and the mean length of the longest neurite extended. Schwann cells evoked a similar response whilst undifferentiated stem cells had no effect. These results indicate adipose tissue contains a pool of regenerative stem cells which can be differentiated to a Schwann cell phenotype and may be of benefit for treatment of peripheral nerve injuries.

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Introduction

Peripheral nerve injuries are a common occurrence and represent a major economic burden for society (Wiberg and Terenghi, 2003). Treatment usually involves direct end–end surgical repair of the damaged nerves for minor defects whereas autologous nerve grafts are required for longer gaps (Lundborg, 2000). Despite good surgical advances, functional recovery is often poor. Tissue engineering techniques which enhance the beneficial endogenous responses to nerve injury could provide an alternative repair strategy.

Schwann cells play a pivotal role in peripheral nerve regeneration (Ide, 1996) and are thus an attractive therapeutic target. Nerve injury disrupts the normal Schwann cell–axon

interaction resulting in dedifferentiation of the Schwann cells and activation of a growth promoting phenotype (Hall, 2005). Proliferating Schwann cells release neurotrophic factors (Frostick et al., 1998) and form the bands of Büngner to direct regenerating axons across the lesion. When seeded in artificial nerve conduits, Schwann cells have been shown to enhance nerve regeneration (Li et al., 2006; Mosahebi et al., 2002; Rutkowski et al., 2004). However, cultured Schwann cells have limited clinical application. The requirement for nerve donor material evokes additional morbidity and the time required to culture and expand the cells would delay treatment. Instead, the ideal transplantable cell should be easily accessible, proliferate rapidly in culture and successfully integrate into host tissue with immunological tolerance (Tohill and Terenghi, 2004).

Mesenchymal stem cells (MSC) are an attractive cell source for the regeneration of nerve tissue as they are able to self-renew with a high growth rate and possess multi-potent differentiation

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properties (Pittenger et al., 1999). There is also some evidence that MSC may be non-immunogenic or hypo-immunogenic (Barry and Murphy, 2004). MSC fibroblast-like cells can be isolated from the stromal cell population found in a number of tissues (Barrilleaux et al., 2006). Bone marrow has been used as the main source of MSC and under appropriate conditions we (Caddick et al., 2006; Tohill et al., 2004) and other groups (Dezawa et al., 2001; Keilhoff et al., 2006) have shown they can be selectively differentiated into Schwann cells. However, the harvest of bone marrow MSC is a highly invasive and painful procedure and alternative sources from which to isolate MSC should be investigated.

In the last few years, adipose tissue has been identified as possessing a population of multi-potent stem cells (Gimble and Guilak, 2003; Strem et al., 2005) to which we assign the generic nomenclature, adipose-derived stem cells (ADSC). The phenotypic and gene expression profiles of ADSC are similar to MSC obtained from bone marrow (De Ugarte et al., 2003a,b; Strem et al., 2005) and these cells can be expanded in culture for extended periods (Zuk et al., 2002). Humans have abundant subcutaneous fat deposits and ADSC can easily be isolated by conventional liposuction procedures, thus overcoming the tissue morbidity associated with bone marrow aspiration. Furthermore the frequency of MSC in bone marrow is between 1 in 25,000 and 1 in 100,000 cells (Banfi et al., 2001; D'Ippolito et al., 1999; Muschler et al., 2001) whereas ADSC constitute approximately 2% of lipoaspirate cells (Strem et al., 2005).

The apparent advantages of ADSC have led us to investigate whether they can be differentiated to a Schwann cell phenotype which could ultimately provide functional benefits for peripheral nerve repair. Animal models are critical to the development of applications utilising ADSC so in this study we have used stem cells isolated from rats. Since rats possess little subcutaneous fat, we used adipose tissue from the abdominal cavity. Previous studies have shown that stem cells isolated from rat visceral fat mimic the differentiation process of human ADSC and can adopt adipocyte, osteoblast, chondrocyte and neural phenotypes (Tholpady et al., 2003).

Materials and methods

Isolation and culture of adipose-derived stem cells (ADSC)

ADSC were isolated from adult Sprague-Dawley rats euthanized by a schedule 1 method according to the UK Animal Scientific Procedures Act 1986. Visceral fat encasing the stomach and intestines was carefully dissected and minced using a sterile razor blade. Tissue was then enzymatically dissociated for 60 min at 37 °C using 0.15% (w/v) collagenase type I (Invitrogen, UK). The solution was passed through a 70- μ m filter to remove undissociated tissue, neutralized by the addition of Modified Eagle Medium (α -MEM; Invitrogen, UK) containing 10% (v/v) foetal bovine serum (FBS) and centrifuged at 800 \times g for 5 min. The stromal cell pellet was resuspended in MEM containing 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin solution. Cultures were maintained at sub-confluent levels in a 37 °C incubator with 5% CO₂ and passaged with trypsin/EDTA (Invitrogen, UK) when required.

Other cell culture

Bone marrow-derived stem cells were harvested from adult rat femoral bones (Caddick et al., 2006) and maintained under the same conditions as ADSC. Schwann cells were isolated from the sciatic nerves of 1- to 2-day-old rat pups (Caddick et al., 2006) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 10 μ M forskolin (Sigma, UK) and 63 ng/ml glial growth factor-2 (GGF-2; Acorda Therapeutics Inc., USA). The NG108-15 cell line was purchased from ECACC (Porton Down, UK) and was maintained in DMEM growth medium.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay

At either passage 1 or passage 5, cells were trypsinised and plated at a density of 5000 cells/35 mm² dish. Cells were allowed to settle for 1 h after which time MTT was added (1 mg/ml final concentration) for a period of 2 h. The resulting formazan precipitate was then solubilised using 20% (v/v) Triton X-100 and the absorbance at 570 nm measured. This value was recorded as the baseline and further measurements were taken every 24 h.

Characterisation of stem cell properties

At passage 1, sub-confluent cultures were treated with agents to induce the phenotype of mesoderm-derived cells. For osteogenic induction, cultures were treated with 100 μ g/ml ascorbate, 0.1 μ M dexamethasone and 10 mM β -glycerophosphate for a period of 3 weeks. Cells were then fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature, washed 3 times with phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin and then incubated with 1% (w/v) Alizarin Red solution to stain for calcium deposition. For induction of a chondrocyte phenotype, cells were treated with 0.1 μ M dexamethasone, 50 μ g/ml ascorbate, 10 ng/ml TGF- β 1, 40 μ g/ml proline and 1% (v/v) ITSTM+premix (BD Biosciences, UK) for 3 weeks. Cells were then fixed with 10% (v/v) formalin for 60 min, washed in H₂O and stained for proteoglycan with 1% (w/v) toluidine blue.

For immunocytochemical assessment of stem cell markers, ADSC were cultured on slide flasks (Nunc-Fisher Scientific, UK) for 24 h and then fixed in 4% (w/v) paraformaldehyde for 20 min. Fixative was removed and cells washed 1 \times 10 min in PBS and permeabilised using 0.2% (v/v) Triton X-100 for 20 min. The cells were washed 2 \times 10 min in PBS and 5% (v/v) normal goat serum blocking solution (Sigma, UK) was added for 1 h at room temperature. Monoclonal stro-1 (1:50; R&D Systems, UK) or nestin (1:500; Chemicon, USA) antibodies were added and incubated at 4 °C overnight. Cells were washed 3 \times 10 min in PBS and goat anti-mouse Cy3-labelled secondary antibody (1:200; Amersham Biosciences, UK) added for 1 h at room temperature. The cells were washed 3 \times 10 min in PBS and slides mounted using an anti-fading Vectashield solution containing DAPI (Vector labs, UK). Slides were examined using an Olympus

IX51 inverted fluorescence microscope and the number of immuno-positive cells counted from a minimum total of 100 cells per experiment.

Differentiation to a Schwann cell phenotype

Growth medium was removed from sub-confluent ADSC cultures at passage 2 and replaced with medium supplemented with 1 mM β -mercaptoethanol (Sigma-Aldrich, UK) for 24 h. Cells were then washed and fresh medium supplemented with 35 ng/ml all-*trans*-retinoic acid was added. A further 72 h later, cells were washed and medium replaced with differentiation medium; cell growth medium supplemented with 5 ng/ml platelet-derived growth factor (PDGF; PeproTech Ltd., UK), 10 ng/ml basic fibroblast growth factor (bFGF; PeproTech Ltd., UK), 14 μ M forskolin and 252 ng/ml GGF-2. Cells were incubated for 2 weeks under these conditions with fresh medium added approximately every 72 h.

Immunocytochemistry and western blotting

Undifferentiated (uADSC) and differentiated (dADSC) cultures were trypsinised and replated on slide flasks for immunostaining as above. Cells were incubated with mouse anti-glial fibrillary acidic protein (GFAP; 1:200; Chemicon, USA), rabbit anti-S100 (1:500; Dako, Denmark) and rabbit anti-p75 (1:500; Promega, USA) overnight at 4 °C. Washed slides were then treated with goat anti-rabbit FITC- (1:100; Vector Labs, UK) and goat anti-mouse CY3 (1:200; Amersham, UK)-conjugated secondary antibodies. Slides were examined using an Olympus IX51 inverted fluorescence microscope and the number of immuno-positive cells counted in a minimum total of 100 cells per experiment.

For western blotting, individual lysates were prepared from one 75-cm² flask of confluent cultures. Cells were washed in PBS and then scraped into buffer containing 100 mM PIPES, 5 mM MgCl₂, 20% (v/v) glycerol, 0.5% (v/v) Triton X-100, 5 mM EGTA and protease inhibitors (Sigma, UK). Lysates were incubated for 15 min on ice and then subjected to 2 freeze–thaw cycles prior to analysis of protein content using a commercially available protein assay kit (Bio-Rad, UK). 15 μ g protein was prepared per sample, combined with Laemmli buffer and denatured at 95 °C for 5 min. Proteins were resolved at 120 V on 15% (for S100) or 10% (for GFAP) sodium dodecyl sulphate–polyacrylamide gels. Following electrophoretic transfer to nitrocellulose, membranes were blocked for 1 h in 5% (w/v) non-fat dry milk in TBS–Tween (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% (v/v) Tween), and then incubated overnight at 4 °C with either monoclonal anti-GFAP (1:200; LabVison, USA), monoclonal anti-S100 (1:750; Chemicon, UK) or polyclonal anti-p75 (1:250; Santa Cruz Biotechnology, USA) antibodies. Following 6 \times 5 min washes in TBS–Tween, membranes were incubated for 1 h with HRP-conjugated secondary antibodies [goat anti-mouse and goat anti-rabbit (1:1000; Cell Signalling Technology, USA)]. Membranes were washed as previously and treated with ECL chemiluminescent substrate (Amersham, UK) for 1 min and developed by ex-

posure to Kodak X-OMAT light-sensitive film. Antibody was stripped from the membranes using 100 mM glycine pH 2.9 and the blots re-probed with β -tubulin antibody (1:1000; Abcam, UK) as a loading control.

Stem cell and NG108-15 neuron co-culture

uADSC and dADSC were plated at a density of 10,000 cells per slide flask and allowed to settle for 24 h. NG108-15 cells were then added to the ADSC monolayer at a density of 1000 cells and the co-cultures maintained for a further 24 h followed by fixation with 4% (w/v) paraformaldehyde for 20 min at room temperature. Fluorescent immunocytochemistry (as above) using mouse anti-neurofilament protein antibody (1:500; BioMol, UK) incubated at 4 °C overnight followed by secondary labelling with goat anti-mouse Cy3 conjugate (1:200; Amersham Biosciences, UK) was used to visualise NG108-15 neurite outgrowth on the ADSC. Co-culture with NG108-15 cells did not alter the differentiation status of ADSC (as measured by expression of glial protein markers). Cultures were examined using an Olympus IX51 inverted fluorescence microscope and images captured using Image ProPlus software (MediaCybernetics, Marlow, UK). The trace function was used to determine the percentage of NG108-15 cells expressing

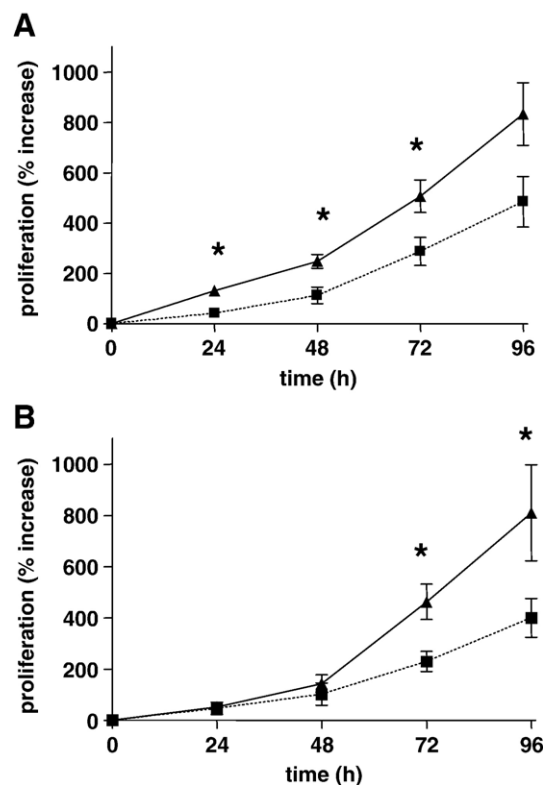


Fig. 1. Proliferation of cells isolated from adipose tissue and bone marrow. An MTT assay was used to determine the growth rate of cells obtained from adipose tissue (\blacktriangle) and bone (\blacksquare) plated at passage 1 (A) and passage 5 (B). Data are expressed as the mean % increase \pm S.E.M. in absorbance (570 nm). * $P < 0.05$ significantly higher in adipose tissue derived cultures compared with those from bone.

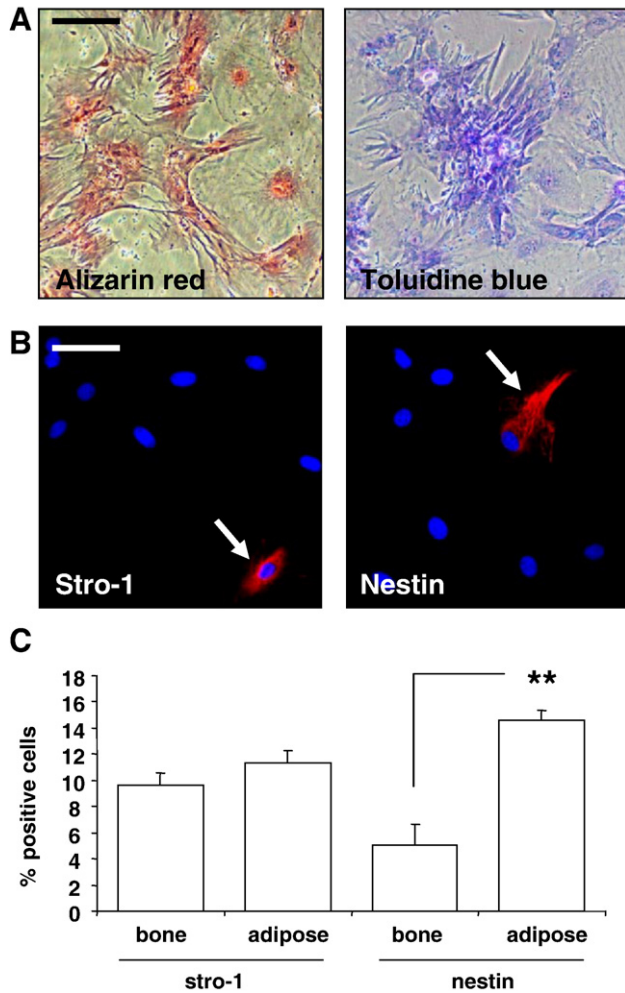


Fig. 2. Adipose tissue-derived cells exhibit properties of stem cells. (A) Cultures were treated with agents to induce differentiation to cells of mesoderm origin. Alizarin red-stained and toluidine blue-stained cells indicate cells of osteogenic and chondrogenic lineages, respectively. Scale bar=40 μ m. (B) Adipose tissue-derived cells at passage 1 were stained with anti-stro-1 and anti-nestin antibodies and CY3-conjugated secondary antibody (red). DAPI staining (blue) indicates the total number of cells in the field and it was used to quantify the percentage of cells positive for each antigen. Scale bar=40 μ m. (C) Data are expressed as the mean % positive \pm S.E.M. ** $P < 0.01$ significantly higher levels of nestin-positive cells in adipose tissue-derived cultures compared with those from bone.

neurites and the number of neurites expressed per cell. For each experiment neurite data were ordered according to length (μ m). The longest neurite in each experiment was thus identified and the mean of these calculated from 5 independent experiments. An average of one hundred NG108-15 cell bodies was analysed for each condition in each experiment.

Statistical analysis

Data are presented as mean \pm S.E.M. from 4 to 5 independent cell cultures. Kruskal–Wallis one way ANOVA with Dunn's comparison test was used to determine the statistical significance between data, * $p < 0.05$; ** $p < 0.01$.

Results

Characterisation of stem cell cultures

Rat visceral adipose tissue was enzymatically digested and then centrifuged to isolate the stromal cell fraction from mature adipocytes. After approximately 1 week in culture, cells from the stromal fraction formed confluent fibroblast-like monolayers on 75-cm² flasks. Cells were then trypsinised and plated for MTT proliferation assays (Fig. 1). There was an apparent lag phase in growth of cells up to 48 h after which time the rate of proliferation expanded more rapidly. Cells isolated from

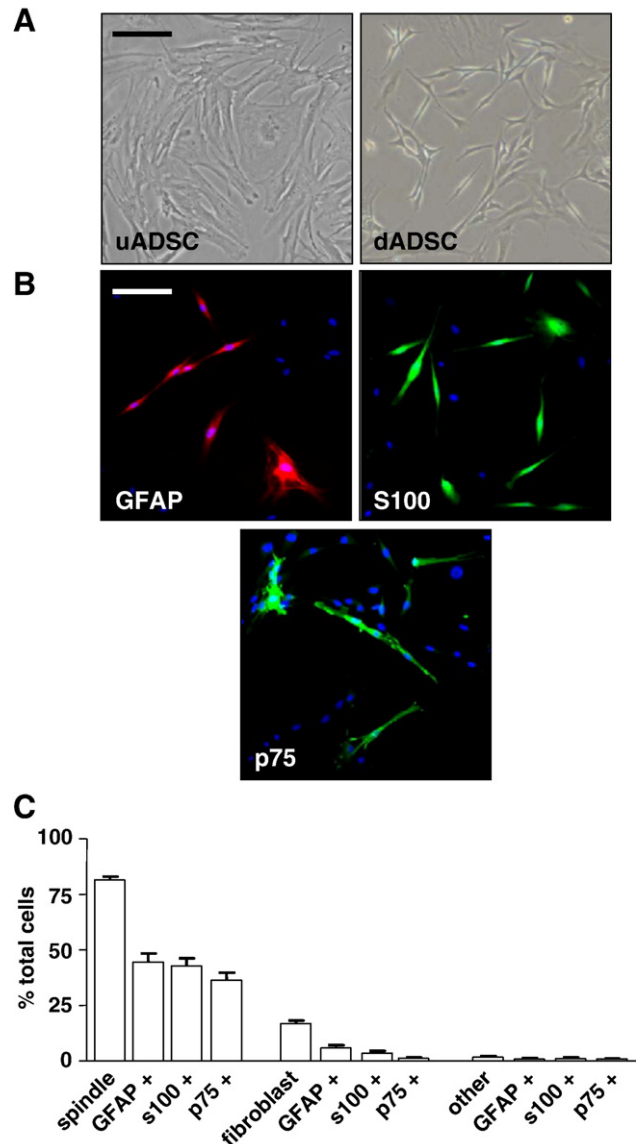


Fig. 3. Adipose-derived stem cells differentiate to a Schwann cell phenotype. (A) Cultures of undifferentiated ADSC (uADSC) showed a flattened fibroblast-like morphology which adopt a spindle elongated shape characteristic of Schwann cells (dADSC) upon treatment with glial growth factors. Scale bar=40 μ m. (B) Immunofluorescence staining indicated differentiated ADSC (dADSC) expressed GFAP, S100 and p75 proteins. Scale bar=30 μ m. (C) Quantitative analysis of morphology and expression of GFAP, S100 and p75 proteins (data are mean % cells \pm S.E.M.).

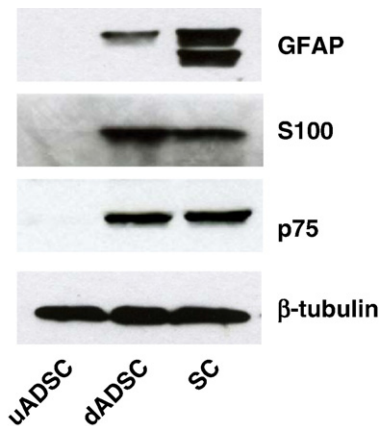


Fig. 4. Western blot analysis of Schwann cell proteins. Undifferentiated ADSC (uADSC), differentiated ADSC (dADSC) and Schwann cell (SC) lysates were blotted for GFAP, S100 and p75 proteins. β -Tubulin was used as a loading control.

bone marrow exhibited a similar growth pattern; however, the overall proliferation rate of cells taken from adipose tissue was significantly faster in passage 1 cultures (Fig. 1A). Although the growth rate of passage 5 adipose cells in the lag phase was not different from bone-derived cells, they still proliferated significantly faster after 48 h (Fig. 1B).

In order to determine whether the cells isolated from adipose tissue exhibited properties of mesenchymal stem cells, they were treated with agents known to induce differentiation to cells originating from the mesoderm. Osteogenic differentiation was confirmed by the production of calcium deposits detected with Alizarin Red (Fig. 2A) and chondrocyte differentiation by the presence of toluidine blue-positive proteoglycans (Fig. 2A). We also examined the passage 1 cultures for the presence of the stem cell marker, *stro-1*. A small proportion ($11.38 \pm 0.87\%$) of cells were positive for *stro-1* in adipose cell cultures, a similar number to that found in bone marrow cultures (Figs. 2B and C). Since we were interested in deriving cells of a neural lineage we also determined whether the cells expressed nestin, a putative marker of neural progenitors (Dahlstrand et al., 1995). There were approximately three times more nestin-positive cells in cultures of cells taken from adipose tissue compared with those from bone (Fig. 2C; $14.6 \pm 0.8\%$ vs. $5.1 \pm 1.6\%$, $P < 0.01$).

Differentiation to a Schwann cell phenotype

Having determined our cultures contained a population of stem-like cells, we assign the term adipose-derived stem cell (ADSC) to describe them. ADSC at passage 2 were treated with a mixture of glial growth factors for a period of 2 weeks after which time they were analysed morphologically and for the expression of the Schwann cell proteins, GFAP, S100 and p75. Cells cultured in the differentiation media changed from a fibroblast-like morphology to an elongated spindle shape (Fig. 3A), similar to that of Schwann cells. Neither GFAP nor S100 protein was detected in undifferentiated cultures (uADSC) (data not shown) but both were expressed in differentiated (dADSC) cells (Fig. 3B). Quantitative analysis indicated that $81.5 \pm 1.5\%$ of the cells adopted a spindle-like morphology of which $44.5 \pm 3.7\%$

expressed GFAP (Fig. 3C). Almost all of these GFAP-positive cells also stained for S100 protein ($42.9 \pm 3.3\%$ positive). p75 expression was occasionally observed in uADSC but was readily apparent in the cultures treated with glial growth factors ($36.38 \pm 3.3\%$ positive). A small fraction ($16.8 \pm 1.4\%$) of treated ADSC retained a fibroblast-like morphology, some of which expressed GFAP ($5.9 \pm 1.3\%$), S100 ($3.4 \pm 1.0\%$) and p75 ($1.23 \pm 0.38\%$) proteins (Fig. 3C). A minority of cells ($1.7 \pm 0.5\%$) displayed a rounded cell body with multiple processes.

To confirm the results obtained by immunocytochemistry were not due to an artefact of cellular shrinkage, western blotting was performed (Fig. 4). Lysates of dADSC but not uADSC showed a GFAP-immunoreactive band corresponding to a molecular weight of 55 kDa. This was present in Schwann cell lysates together with an additional lower band which is likely to represent a proteolytic fragment or alternate transcript.

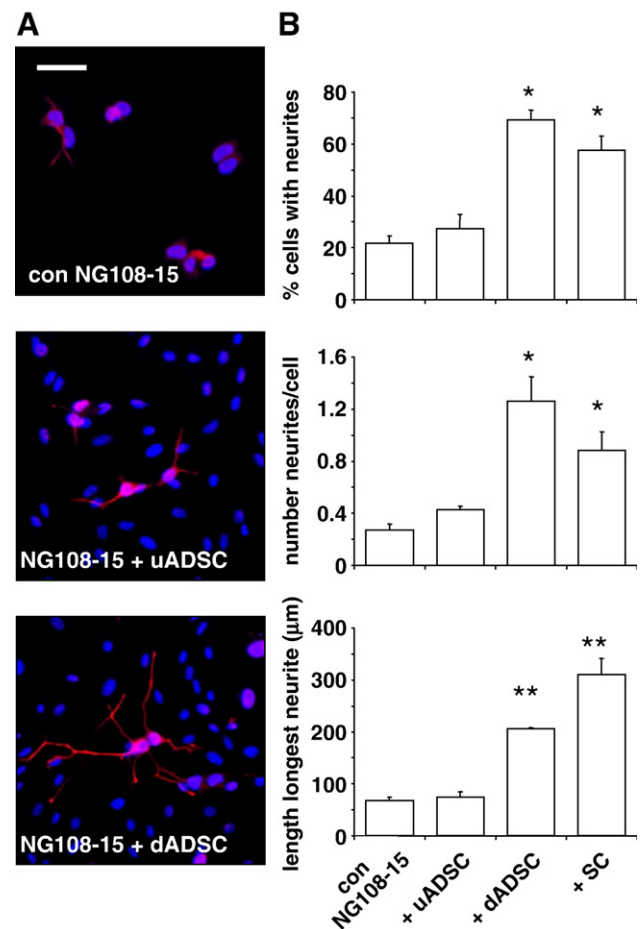


Fig. 5. Differentiated ADSC promote neurite outgrowth in NG108-15 cells. (A) NG108-15 cells were grown alone (con NG108-15) or on a monolayer of undifferentiated (NG108-15+uADSC) or differentiated (NG108-15+dADSC) stem cells. Cultures were stained with neurofilament antibody (red) to visualise neurites and DAPI (blue) to highlight individual cells. Scale bar=60 μ m. (B) The percentage of NG108-15 cells expressing neurites, number of neurites per cell and length of longest neurite were determined in NG108-15 cells grown alone (con NG108-15) and co-cultures with stem cells (+uADSC, +dADSC) and Schwann cells (+SC). Data are mean \pm S.E.M. * $P < 0.05$; ** $P < 0.01$ significantly different compared with NG108-15 grown alone.

S100 and p75 proteins were also detected in dADSC but were absent in uADSC.

Functional properties of differentiated cells

The ability of ADSC to promote neurite outgrowth was determined by examining their interaction with NG108-15 cells, a motor neuron-like cell line (Jiang et al., 2003). uADSC and dADSC were plated on slide flasks to form monolayers and then NG108-15 cells were added (Fig. 5A). Computerised image analysis of co-cultures after 24 h was used to quantify three separate parameters: percentage of cells extending neurites, number of neurites per cell and length of longest neurite (Fig. 5B). Comparisons were made with control cultures of NG108-15 cells grown alone and NG108-15 cells seeded with Schwann cells. A small fraction ($22.0 \pm 2.5\%$) of control NG108-15 cells extended neurites which was significantly increased to $69.1 \pm 4.1\%$ ($P < 0.05$) and $57.6 \pm 5.3\%$ ($P < 0.05$) in the presence of dADSC and Schwann cells respectively. uADSC had no significant effect. Likewise the number of neurites extended per cell was significantly ($P < 0.05$) increased in co-cultures of NG108-15 cells with dADSC or Schwann cells, when compared with NG108-15 cells grown alone. The mean longest neurite extended by control cultures of NG108-15 cells was $67.5 \pm 7.5 \mu\text{m}$ and in co-culture with uADSC it was $74.3 \pm 9.7 \mu\text{m}$. In contrast, dADSC evoked a significant ($P < 0.01$) increase in neurite length to $205.2 \pm 2.7 \mu\text{m}$ and Schwann cells stimulated an increase to $309.8 \pm 31.5 \mu\text{m}$ ($P < 0.01$).

Discussion

We have recently shown that MSC derived from bone marrow can undergo differentiation to a Schwann cell phenotype (Caddick et al., 2006; Tohill et al., 2004). Given the clinical advantages of adipose tissue as an alternative source of stem cells (Gimble and Guilak, 2003; Strem et al., 2005), we have now investigated whether it is also possible to derive Schwann cells from adipose tissue. We found that rat ADSC treated with a mixture of glial growth factors expressed GFAP, S100 and p75 proteins and enhanced neurite outgrowth in vitro, suggesting transition to a Schwann cell phenotype.

As part of an initial characterisation of our cultures, we compared the growth rate of cells isolated from adipose tissue and bone marrow. We found that ADSC proliferated significantly faster than bone MSC. This is consistent with a recent study comparing various sources of rat MSC (Yoshimura et al., 2007) and if translated to human studies could mean a reduction in the time required to generate a therapeutically useful stock of cells. In order to determine the “stem-ness” of our cells we investigated the expression of *stro-1*. In contrast to the study by Ning et al. (2006) which identified all ADSC as *stro-1* positive, only a small fraction of our cultures expressed this marker. This apparent discrepancy has also been reported by different groups examining human ADSC (De Ugarte et al., 2003a,b; Gronthos et al., 2001) and might reflect a difference in the region from which the tissue was obtained.

Nestin is a protein commonly used to identify proliferating adult neural progenitor cells in the central nervous system

(Dahlstrand et al., 1995). More recently nestin expression has been observed in bone marrow MSC (Caddick et al., 2006; Wislet-Gendebien et al., 2004) and its importance in controlling commitment to differentiation along the glial lineage has been demonstrated (Wislet-Gendebien et al., 2004). Our results showed that when compared with bone marrow MSC, a significantly greater proportion of ADSC expressed nestin protein. Murine ADSC have also been shown to express low levels of nestin which can be up-regulated upon neurogenic differentiation (Safford et al., 2002). These results suggest that ADSC are not restricted towards specific mesodermal cell lineages and rather they retain some ability for differentiation along a neuroglial lineage.

To investigate this, we exposed the ADSC to a differentiation media we have previously used to induce Schwann cells from bone marrow MSC (Caddick et al., 2006). This produced a morphological change in the majority of the cells, toward an elongating spindle phenotype, characteristic of Schwann cells. Many of these cells also began to express the glial markers, GFAP, S100 and p75. The co-expression of these proteins taken with the morphological changes indicates we can produce high yields of Schwann-like cells from ADSC. In contrast, previous reports have shown that neural induction media converts ADSC to cells of a neuronal morphology with co-expression of GFAP, S100 and neuronal proteins including β III-tubulin and neurofilament (Ning et al., 2006; Safford et al., 2004). Krampera et al. (2007) recently showed these changes were rapid and reversible, suggesting against a specific, full differentiation process. To induce a more selective Schwann cell differentiation, the authors co-cultured various MSC with Schwann cells and this produced a long lasting expression of PMP-20 and S100 proteins in the absence of other CNS glial and neuronal markers (Krampera et al., 2007). Interestingly, of all the MSC tested, those derived from adipose tissue produced the best response. These effects could not be induced by Schwann cell factors alone, indicating contact between the two cell types was necessary (Krampera et al., 2007). Whilst this suggests that some form of *trans*-differentiation might occur if ADSC were transplanted at a nerve injury site in vivo, the methodology does not provide a suitable approach for the generation of clinically useful cells. Instead, we have used a defined mixture of GGF-2, bFGF and PDGF, molecules which are known to play a role in the differentiation and proliferation of Schwann cells (Jessen and Mirsky, 1999; Li et al., 1998). These molecules together with forskolin are responsible for the induction of the glial protein expression we observed by immunocytochemistry. These results were confirmed by western blotting; arguing against the notion suggested by Lu et al. (2004) that increased staining is merely the result of an increase in antigen levels per unit area, due to disruption of the cytoskeleton.

We also tested the function of our differentiated ADSC using a co-culture with the NG108-15 motor neuron-like cell line. We found that differentiated ADSC evoked a similar response to Schwann cells, in that they promoted neurite outgrowth and elongation. NG108-15 cells have been shown to express low levels of *trkA* (Fu et al., 1997) and *GFR- α 1* (Lee et al., 2006), receptors for nerve growth factor (NGF) and glial-derived neurotrophic factor (GDNF), respectively, two proteins which

are known to be released by Schwann cells (Frostick et al., 1998). To date, there have been no reports of the expression of neurotrophic factors produced by ADSC. However, a recent study has shown that rat bone marrow cells constitutively express NGF, GDNF and brain-derived neurotrophic factor (BDNF) (Chen et al., 2007). Human MSC also express NGF and BDNF although this is restricted to certain sub-populations (Crigler et al., 2006). These and other unidentified factors promoted neurite outgrowth and survival in a co-culture system with SH-SY5Y cells (Crigler et al., 2006). In our model, undifferentiated ADSC had no effect on neurite outgrowth suggesting that the process of differentiation led to the up-regulation of nerve growth factors. In on-going experiments to identify the factors involved we have determined that conditioned medium from dADSC increases the number of NG108-15 cells producing neurites and neurite number per cell but does not enhance neurite length (data not shown). Thus direct contact (the model used in this study) between the cells is required for neurite elongation and this suggests that dADSC might also up-regulate extracellular matrix molecules such as laminin or fibronectin, which have also been shown to be expressed by rat bone marrow MSC (Chen et al., 2007).

Our results suggest that ADSC differentiated to a Schwann cell phenotype might have a beneficial role for treatment of peripheral nerve injuries. We have previously shown that only differentiated bone marrow MSC, rather than untreated MSC, can stimulate nerve regeneration (Tohill et al., 2004), a result in agreement with other groups (Dezawa et al., 2001; Keilhoff et al., 2006). In contrast, other studies indicate that undifferentiated bone marrow MSC can also enhance regeneration and lead to improved motor function (Chen et al., 2007; Pereira Lopes et al., 2006). This suggests that some form of *trans*-differentiation might occur in vivo as the result of local signals from injured Schwann cells and axons. However, the long-term effect of undifferentiated cells remains to be determined and therefore we will continue to study the mechanisms of differentiation of ADSC, in the hope of generating clinically useful cells, for the treatment of peripheral nerve injuries.

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References

- Banfi, A., Bianchi, G., Galotto, M., Cancedda, R., Quarto, R., 2001. Bone marrow stromal damage after chemo/radiotherapy: occurrence, consequences and possibilities of treatment. *Leuk. Lymphoma* 42, 863–870.
- Barrilleaux, B., Phinney, D.G., Prockop, D.J., O'Connor, K.C., 2006. Review: ex vivo engineering of living tissues with adult stem cells. *Tissue Eng.* 3007–3019.
- Barry, F.P., Murphy, J.M., 2004. Mesenchymal stem cells: clinical applications and biological characterization. *Int. J. Biochem. Cell Biol.* 36, 568–584.
- Caddick, J., Kingham, P.J., Gardiner, N.J., Wiberg, M., Terenghi, G., 2006. Phenotypic and functional characteristics of mesenchymal stem cells differentiated along a Schwann cell lineage. *Glia* 54, 840–849.
- Chen, C.J., Ou, Y.C., Liao, S.L., Chen, W.Y., Chen, S.Y., Wu, C.W., Wang, C.C., Wang, W.Y., Huang, Y.S., Hsu, S.H., 2007. Transplantation of bone marrow stromal cells for peripheral nerve repair. *Exp. Neurol.* 204, 443–453.
- Crigler, L., Robey, R.C., Asawachaicharn, A., Gaupp, D., Phinney, D.G., 2006. Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neurogenesis. *Exp. Neurol.* 198, 54–64.
- D'Ippolito, G., Schiller, P.C., Ricordi, C., Roos, B.A., Howard, G.A., 1999. Age-related osteogenic potential of mesenchymal stromal stem cells from human vertebral bone marrow. *J. Bone Miner. Res.* 14, 1115–1122.
- Dahlstrand, J., Lardelli, M., Lendahl, U., 1995. Nestin mRNA expression correlates with the central nervous system progenitor cell state in many, but not all, regions of developing central nervous system. *Brain Res. Dev. Brain Res.* 84, 109–129.
- De Ugarte, D.A., Alfonso, Z., Zuk, P.A., Elbarbary, A., Zhu, M., Ashjian, P., Benhaim, P., Hedrick, M.H., Fraser, J.K., 2003a. Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. *Immunol. Lett.* 89, 267–270.
- De Ugarte, D.A., Morizono, K., Elbarbary, A., Alfonso, Z., Zuk, P.A., Zhu, M., Drago, J.L., Ashjian, P., Thomas, B., Benhaim, P., Chen, I., Fraser, J., Hedrick, M.H., 2003b. Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* 174, 101–109.
- Dezawa, M., Takahashi, I., Esaki, M., Takano, M., Sawada, H., 2001. Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells. *Eur. J. Neurosci.* 14, 1771–1776.
- Frostick, S.P., Yin, Q., Kemp, G.J., 1998. Schwann cells, neurotrophic factors, and peripheral nerve regeneration. *Microsurgery* 18, 397–405.
- Fu, A.K., Ip, F.C., Lai, K.O., Tsim, K.W., Ip, N.Y., 1997. Muscle-derived neurotrophin-3 increases the aggregation of acetylcholine receptors in neuron–muscle co-cultures. *Neuroreport* 8, 3895–3900.
- Gimble, J., Guilak, F., 2003. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* 5, 362–369.
- Gronthos, S., Franklin, D.M., Leddy, H.A., Robey, P.G., Storms, R.W., Gimble, J.M., 2001. Surface protein characterization of human adipose tissue-derived stromal cells. *J. Cell. Physiol.* 189, 54–63.
- Hall, S., 2005. The response to injury in the peripheral nervous system. *J. Bone Jt. Surg., Br.* 87, 1309–1319.
- Ide, C., 1996. Peripheral nerve regeneration. *Neurosci. Res.* 25, 101–121.
- Jessen, K.R., Mirsky, R., 1999. Developmental regulation in the Schwann cell lineage. *Adv. Exp. Med. Biol.* 468, 3–12.
- Jiang, J.X., Choi, R.C., Siow, N.L., Lee, H.H., Wan, D.C., Tsim, K.W., 2003. Muscle induces neuronal expression of acetylcholinesterase in neuron–muscle co-culture: transcriptional regulation mediated by cAMP-dependent signaling. *J. Biol. Chem.* 278, 45435–45444.
- Keilhoff, G., Gohl, A., Langnese, K., Fansa, H., Wolf, G., 2006. Transdifferentiation of mesenchymal stem cells into Schwann cell-like myelinating cells. *Eur. J. Cell Biol.* 85, 11–24.
- Krampera, M., Marconi, S., Pasini, A., Galie, M., Rigotti, G., Mosna, F., Tinelli, M., Lovato, L., Anghileri, E., Andreini, A., Pizzolo, G., Sbarbati, A., Bonetti, B., 2007. Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. *Bone* 40, 382–390.
- Lee, R.H., Wong, W.L., Chan, C.H., Chan, S.Y., 2006. Differential effects of glial cell line-derived neurotrophic factor and neurturin in RET/GFRalpha1-expressing cells. *J. Neurosci. Res.* 83, 80–90.
- Li, H., Wigley, C., Hall, S.M., 1998. Chronically denervated rat Schwann cells respond to GGF in vitro. *Glia* 24, 290–303.
- Li, Q., Ping, P., Jiang, H., Liu, K., 2006. Nerve conduit filled with GDNF gene-modified Schwann cells enhances regeneration of the peripheral nerve. *Microsurgery* 26, 116–121.
- Lu, P., Blesch, A., Tuszynski, M.H., 2004. Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? *J. Neurosci. Res.* 77, 174–191.

- Lundborg, G., 2000. A 25-year perspective of peripheral nerve surgery: evolving neuroscientific concepts and clinical significance. *J. Hand Surg. [Am.]* 25, 391–414.
- Mosahebi, A., Fuller, P., Wiberg, M., Terenghi, G., 2002. Effect of allogeneic Schwann cell transplantation on peripheral nerve regeneration. *Exp. Neurol.* 173, 213–223.
- Muschler, G.F., Nitto, H., Boehm, C.A., Easley, K.A., 2001. Age- and gender-related changes in the cellularity of human bone marrow and the prevalence of osteoblastic progenitors. *J. Orthop. Res.* 19, 117–125.
- Ning, H., Lin, G., Lue, T.F., Lin, C.S., 2006. Neuron-like differentiation of adipose tissue-derived stromal cells and vascular smooth muscle cells. *Differentiation* 74, 510–518.
- Pereira Lopes, F.R., Camargo de Moura, C.L., Dias Jr., C.J., Balduino, A., Lora, S., Langone, F., Borojevic, R., Blanco Martinez, A.M., 2006. Bone marrow stromal cells and resorbable collagen guidance tubes enhance sciatic nerve regeneration in mice. *Exp. Neurol.* 198, 457–468.
- Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., Marshak, D.R., 1999. Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–147.
- Rutkowski, G.E., Miller, C.A., Jeftinija, S., Mallapragada, S.K., 2004. Synergistic effects of micropatterned biodegradable conduits and Schwann cells on sciatic nerve regeneration. *J. Neural Eng.* 1, 151–157.
- Safford, K.M., Hicok, K.C., Safford, S.D., Halvorsen, Y.D., Wilkison, W.O., Gimble, J.M., Rice, H.E., 2002. Neurogenic differentiation of murine and human adipose-derived stromal cells. *Biochem. Biophys. Res. Commun.* 294, 371–379.
- Safford, K.M., Safford, S.D., Gimble, J.M., Shetty, A.K., Rice, H.E., 2004. Characterization of neuronal/glial differentiation of murine adipose-derived adult stromal cells. *Exp. Neurol.* 187, 319–328.
- Strem, B.M., Hicok, K.C., Zhu, M., Wulur, I., Alfonso, Z., Schreiber, R.E., Fraser, J.K., Hedrick, M.H., 2005. Multipotential differentiation of adipose tissue-derived stem cells. *Keio J. Med.* 54, 132–141.
- Tholpady, S.S., Katz, A.J., Ogle, R.C., 2003. Mesenchymal stem cells from rat visceral fat exhibit multipotential differentiation in vitro. *Anat. Rec., A Discov. Mol. Cell Evol. Biol.* 272, 398–402.
- Tohill, M., Terenghi, G., 2004. Stem-cell plasticity and therapy for injuries of the peripheral nervous system. *Biotechnol. Appl. Biochem.* 40, 17–24.
- Tohill, M., Mantovani, C., Wiberg, M., Terenghi, G., 2004. Rat bone marrow mesenchymal stem cells express glial markers and stimulate nerve regeneration. *Neurosci. Lett.* 362, 200–203.
- Wiberg, M., Terenghi, G., 2003. Will it be possible to produce peripheral nerves? *Surg. Technol. Int.* 11, 303–310.
- Wislet-Gendebien, S., Bruyere, F., Hans, G., Leprince, P., Moonen, G., Rogister, B., 2004. Nestin-positive mesenchymal stem cells favour the astroglial lineage in neural progenitors and stem cells by releasing active BMP4. *BMC Neurosci.* 5, 33.
- Yoshimura, H., Muneta, T., Nimura, A., Yokoyama, A., Koga, H., Sekiya, I., 2007. Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res.* 327, 449–462.
- Zuk, P.A., Zhu, M., Ashjian, P., De Ugarte, D.A., Huang, J.I., Mizuno, H., Alfonso, Z.C., Fraser, J.K., Benhaim, P., Hedrick, M.H., 2002. Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell* 13, 4279–4295.